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Detailed protocols for each of the tests listed below are available for review in Appendix F, which contains Standard Operating Procedures (SOP's) for the entities conducting Toxicity Testing for SWAMP. The summaries provided below pertain to those tests and protocols conducted by staff of the U.C. Davis Department of Environmental Toxicology at the Granite Canyon Marine Laboratory, south of Monterey, California.

- **Marine and Estuarine Amphipod Acute Survival Tests (solid-phase sediment--saltwater)**

Acute toxicity in solid-phase sediment samples is assessed using the 10-day amphipod survival toxicity test protocols described in EPA 2000. Four species can be used with this protocol, depending on salinity, grain size, and other considerations: *Eohaustorius estuarius, Rhepoxynius abronius, Ampelisca abdita*, and *Grandidierella japonica*. *Eohaustorius* will likely be the most commonly used species in the SWAMP program.

Upon arrival at Granite Canyon, the *Eohaustorius* are acclimated to 20‰ (T=15°C), and *Rhepoxynius* are acclimated to 28‰ (T=15°C). Once acclimated, the animals are held for an additional 48-hours prior to addition to the test containers. Upon arrival at Granite Canyon, the *Ampelisca* are acclimated slowly (<2‰ per day) to 28‰ seawater (T=20°C). Once acclimated, the animals are held for an additional 48 hours prior to inoculation into the test containers. Test containers are one-liter glass beakers or jars containing 2 cm of sediment and filled to the 700-ml line with control seawater adjusted to the appropriate salinity using spring water or distilled well water. Test sediments are not sieved for indigenous organisms prior to testing unless they contain *Ampelisca*. At the conclusion of the test, the presence of any predators are noted and recorded on the data sheet. Test sediment and overlying water are allowed to equilibrate for 24 hours, after which 20 amphipods are placed in each beaker along with control seawater to fill test containers to the one-liter line. Test chambers are aerated gently and illuminated continuously at ambient laboratory light levels. Five laboratory replicates of each sample are tested for ten days. A negative sediment control consisting of five lab replicates of collection site home sediment are included with each sediment test. After ten days, the sediments are sieved through a 400 or 500 μm Nitex screen to recover the test animals, and the number of survivors is recorded for each replicate.

- ***Atherinops affinis* 7-Day Survival and Growth Test (saltwater)**

Aquatic toxicity of marine water samples is assessed using the topsmelt (*Atherinops affinis*) growth and survival test. This 7-day method follows US EPA 1995. Nine- to fourteen-day-old fish are purchased from an organism supplier and acclimated for 2 days in natural seawater at 20°C. Five fish are randomly distributed to test containers containing 200 mL of sample. Test containers are checked daily and the number of living fish is recorded. Immobile fish that do not respond to a stimulus are considered dead. Fish are fed 40 freshly hatched *Artemia* nauplii per fish twice daily. Test solutions are 50% renewed at 48 and 96 hours. The laboratory negative control consists of Granite Canyon seawater filtered to one micron. A positive control reference test is conducted

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concurrently with the test using a dilution series of copper chloride as the reference toxicant.

- ***Ceriodaphnia dubia* 96-hour Survival Tests (freshwater)**

Ceriodaphnia dubia 96-hour toxicity tests will be conducted on water samples using US EPA standard test protocols (US EPA 1993). Each undiluted sample is tested using 5 replicates. Each replicate contains 5 *Ceriodaphnia* neonates (< 24-h-old). Survival is monitored daily in each replicate of each sample. Water quality parameters including conductivity, hardness, alkalinity, pH, dissolved oxygen, and ammonia is measured in one replicate of each sample at the beginning of each test. Dissolved oxygen, conductivity, and pH are measured in one replicate of each old and new test solution at the 48-hour renewal, and at test termination. Ammonia is measured at renewal and termination if initial measurements are above ½ the LC_{50}. Temperature is monitored continuously by placing a temperature probe in an additional test solution in the controlled temperature room.

- ***Ceriodaphnia dubia* 7-Day Survival and Reproduction Tests (freshwater)**

Ceriodaphnia dubia 7-day toxicity tests are conducted on water samples using US EPA standard test protocols (US EPA 1994). Each undiluted sample is tested using 10 replicates. Each replicate contains one *Ceriodaphnia* neonate (< 24-h-old). Survival and reproduction are monitored daily in each replicate of each sample. Water quality parameters including conductivity, hardness, alkalinity, pH, dissolved oxygen, and ammonia are measured at the beginning of each test. Test solutions are renewed daily and dissolved oxygen and pH are measured on the old solution. Dissolved oxygen is measured on the new solution. Temperature is monitored continuously by placing a temperature probe in an additional test solution in the controlled temperature room.

- ***Haliotis rufescens* Abalone Embryo-Larval Development Test (saltwater)**

The red abalone (*Haliotis rufescens*) embryo-larval development test is conducted on porewater and water samples. Details of the test protocol are given in US EPA 1995. Adult male and female abalone are induced to spawn separately using a dilute solution of hydrogen peroxide in seawater. Fertilized eggs are distributed to the test containers within one hour of fertilization. Test containers are generally polyethylene-capped, seawater leached, 20-ml glass scintillation vials containing 10 mL of sample. Each test container is inoculated with 100 embryos (10/mL). Controls consist of Granite Canyon seawater, and a brine control is included with all samples that require brine adjustment. Tests are conducted at ambient seawater salinity (33±2‰). A 48-h positive control reference test is conducted concurrently with each test using a dilution series of zinc sulfate as a reference toxicant. After a 48-h exposure period, developing larvae are fixed in 5% buffered formalin. All larvae in each container are examined using an inverted light microscope at 100x to determine the proportion of veliger larvae with normal shells, as described in US EPA 1995.

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- ***Holmesimysis costata* Mysid Survival and Growth Test (saltwater)**

Toxicity of marine water samples is assessed using the mysid (*Holmesimysis costata*) 7-day growth and survival test (US EPA 1995).. The mysid shrimp, *Holmesimysis costata*, commonly inhabits the surface canopy of the giant kelp *Macrocystis pyrifera*. Mysids are collected approximately 7 days prior to test initiation. Females carrying eyed embryos are placed in brood chambers within holding tanks. Juvenile mysids released over a twenty-four hour period are isolated and transferred to a separate tank. Three to four day-old juveniles are randomly distributed to test containers containing 200 mL of sample. Each container receives five mysids. Test containers are checked daily and the number of living mysids is recorded. Immobile mysids that do not respond to a stimulus are considered dead. Mysids are fed 20 freshly hatched *Artemia* nauplii per mysid twice daily. Fifty percent of the test solution is renewed at 48 and 96 hours. The laboratory negative control consists of Granite Canyon seawater filtered to one micron. A positive control reference test is conducted concurrently with the test using a dilution series of zinc sulfate as the reference toxicant.

- ***Hyalella azteca* 10-day Acute Survival and Growth Test (solid-phase sediments: freshwater)**

The toxicity of freshwater sediment is assessed using the *Hyalella azteca* 10-day growth and survival test following EPA standard protocols (US EPA 2000). Each sediment sample is tested with 8 replicates of 10 *Hyalella* individuals each, with growth and survival recorded on day 10. MPSL well water is used as overlying water for each sediment sample. Water quality parameters, including conductivity, hardness, alkalinity, pH, dissolved oxygen, and ammonia are measured in one replicate of each sample at the beginning and end of each sediment test. Dissolved oxygen is measured daily in one replicate of each sediment sample. Temperature is monitored continuously by placing a probe in an additional test solution in the controlled temperature room.

- ***Hyalella azteca* 28-day Chronic Growth and Survival Toxicity Test (solid-phase sediments: freshwater)**

The chronic toxicity of freshwater sediments is assessed using the *Hyalella azteca* 28-day growth and survival test (US EPA 2000). All methods for this test are identical to those for the 10-day test, the only difference being test duration.

- ***Macrocystis pyrifera* Germination and Growth Test**

The toxicity of marine water samples can be assessed using the giant kelp (*Macrocystis pyrifera*) germination and growth test (EPA 1995). Reproductive blades of the plants (sporophylls) are collected the day before test initiation and stored overnight at 9-12°C. Prior to test initiation, blades are desiccated and spores are released. Approximately 2 mL of spore solution are added to 200 mL test solution for a final spore density of 7500 spores/mL. Test containers are glass crystallizing dishes or similar plastic containers containing a standard microscope slide. Controls include a Granite Canyon seawater control plus a brine control for all samples that require salinity adjustment. Tests are

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conducted at 34 ± 2‰. A 48-h positive control reference test is conducted concurrently with each test using a dilution series of copper chloride as a reference toxicant. After a 48-h exposure, slides are removed, covered and blotted of excess water. Slides are examined using a light microscope at 400x to determine the proportion of germinated spores and the length of 10 germ tubes.

- ***Mytilus* spp. Embryo-Larval Development Test (saltwater)**

The toxicity of marine and estuarine waters and porewaters can be assessed using the bay mussel (*Mytilus* spp.) embryo-larval development test (EPA 1995)... Adult male and female mussels are induced to spawn separately using a temperature shock that raises the ambient temperature by 10°C. Fertilized eggs are distributed to the test containers within four hours of fertilization. Test containers are polyethylene-capped, seawater leached, 20-ml glass scintillation vials containing 10 mL of sample. Each test container is inoculated with 150 to 300 embryos (15-30/mL), with less than 20% variation in innoculation density among replicates and treatments within a test set. Controls include a Granite Canyon seawater control plus a brine control for all samples that require salinity adjustment. Tests are conducted at 28 or 34 ± 2‰. A 48-h positive control reference test is conducted concurrently with each test using a dilution series of cadmium chloride as a reference toxicant. After a 48-h exposure period, developing larvae are fixed in 5% buffered formalin. All larvae in each container are examined using an inverted light microscope at 100x to determine the proportion of normal live prossidoconch larvae, as described in EPA 1995.

- ***Pimephales promelas* 7-Day Survival and Growth Test (freshwater)**

The toxicity of freshwater samples can be assessed using the fathead minnow (*Pimephales promelas*) growth and survival test (US EPA 1994). Less-than 24-hour old fish are purchased from an organism supplier and received on the day the test is initiated. Ten fish are randomly distributed to test containers containing 250 mL of sample. Test containers are checked daily and the number of living fish is recorded. Immobile fish that do not respond to a stimulus are considered dead. Fish are fed freshly hatched *Artemia* nauplii twice daily. Water quality parameters including conductivity, hardness, alkalinity, pH, dissolved oxygen, and ammonia are measured at the beginning of each test. Test solutions are renewed daily and dissolved oxygen and pH are measured on the old solution. Dissolved oxygen is measured on the new solution. Temperature is monitored continuously by placing a temperature probe in an additional test solution in the controlled temperature room.

- ***Selenastrum capricornutum* 96-Hour Growth Test (freshwater)**

The toxicity of freshwater samples can be assessed using the single cell alga *Selenastrum capricornutum* growth test (US EPA 1994). . An algal culture that is 4 to 7 days old is used to inoculate test containers containing 25 to 50 mL sample. Containers are incubated at 25°C for four days and shaken twice daily. At the termination of the test the number of cells per mL are determined. Water quality parameters including conductivity,

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hardness, alkalinity, pH, dissolved oxygen, and ammonia are measured at the beginning of each test. pH is measured daily. Temperature is monitored continuously by placing a temperature probe in an additional test solution in the controlled temperature room.

- ***Strongylocentrotus purpuratus* Sea Urchin Embryo-Larval Development Test (saltwater)**

The toxicity of marine and estuarine waters and porewaters can be assessed using the sea urchin (*Strongylocentrotus purpuratus*) larval development test (EPA 1995). Sea urchins are collected from the Monterey County coast near Granite Canyon, and held at MPSL at ambient seawater temperature and salinity (33±2‰) until testing. Adult sea urchins are held in complete darkness to preserve gonadal condition. On the day of a test, urchins are induced to spawn in air by injection with 0.5M KCl. Eggs and sperm collected from the urchins are mixed in seawater at a 500 to 1 sperm to egg ratio, and embryos are distributed to test containers within 1 hour of fertilization. Test containers are polyethylene-capped, seawater leached, 20-ml glass scintillation vials containing 10 mL of sample. Each test container is inoculated with approximately 250 embryos (25/ml). Controls include a Granite Canyon seawater control plus a brine control for all samples that require salinity adjustment.. Tests are conducted at ambient seawater salinity (33±2‰). A 96-hour positive control reference test is conducted concurrently with each test using a dilution series of copper chloride as a reference toxicant. After a 96-hour exposure, larvae are fixed in 5% buffered formalin. Approximately 100 larvae in each container are examined under an inverted light microscope at 100x to determine the proportion of normally developed larvae as described in EPA 1995. Visual clues used to identify embryos as normal included development of skeletal rods (spicules) that extend beyond half the length of the larvae and normal development of a three-part gut. Embryos demonstrating retarded development are considered abnormal.

- ***Strongylocentrotus purpuratus* Sea Urchin Fertilization Test (saltwater)**

The toxicity of marine and estuarine waters and porewaters can be assessed using the sea urchin (*Strongylocentrotus purpuratus*) fertilization test (US EPA 1995). On the day of a test, urchins are induced to spawn in air by injection with 0.5M KCl. Sperm are exposed to sample in test containers for 20 minutes before approximately 1000 eggs are added. After twenty minutes of fertilization, the test is fixed in a 5% buffered formalin solution. Fertilization is determined by the presence or absence of a fertilization membrane. Test containers are polyethylene-capped, seawater leached, 20-ml glass scintillation vials containing 5 mL of pore water. Controls include a Granite Canyon seawater control plus a brine control for all samples that require salinity adjustment.. Tests are conducted at ambient seawater salinity (33±2 ppt). A positive control reference test is conducted concurrently with each test using a dilution series of copper chloride as a reference toxicant.

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Sediment-Water Interface Exposure System

Sediment toxicity can be assessed by exposing various species at early life stages in sediment-water interface exposure systems. Intact sediment core samples are taken with minimal disturbance directly from the substrate or from a Van Veen grab sampler. Details of the test protocols are given in the MPSL Standard Operating Procedure, which follows the EPA methods manual (1995). Sediment-water interface test containers consist of a polycarbonate tube with a 25-µm screened bottom placed so that the screen is within 1 cm of the surface of an intact sediment core (Anderson et al. 1996). Dilution water is poured into the core tube and allowed to equilibrate for 24 hours before the start of the test. After inserting the screen tube into the equilibrated cores, each tube is inoculated with test test organisms. The laboratory control consists of reference sediment. A positive control reference test using a toxicant dilution series is conducted concurrently.

ELISA Tests

Water and porewater samples can be analyzed for diazinon and chlorpyrifos at MPSL using enzyme-linked immunosorbent assays (ELISAs). In this method, polyclonal antibodies that bind to both the chemical of interest (diazinon or chlorpyrifos) and enzyme conjugates of known concentrations are immobilized to the walls of test wells. Both the test sample and the enzyme conjugate are added to the test wells. The chemical of interest and the enzyme conjugate are allowed to compete for the limited number of antibody binding sites. A colorless substrate solution is then added to the test well. The substrate turns blue in the presence of bound enzyme conjugate. A sample that contains a low concentration of the chemical of interest allows the antibody to bind many enzyme-conjugate molecules, resulting in a dark blue solution. Color is inversely proportional to the concentration of the chemical of interest, and is measured with a spectrophotometer. A 5-point standard curve is constructed using a chemical standard provided by the manufacturer.

Samples will be analyzed with ELISA at full strength, unless initial readings indicate that the chemical is at concentrations above the range of the test kits. In such cases, samples will be diluted prior to definitive analysis. The lowest detectable doses are 30 ng/L for diazinon and 50 ng/L for chlorpyrifos (Sullivan and Goh 2000).

Toxicity Identification Evaluation (TIE) Procedures

Phase I TIEs are designed to characterize broad classes of compounds responsible for observed toxicity. Phase I TIE procedures include adjustment of sample pH, chelation of cationic compounds (including many trace metals), neutralization of oxidants (such as chlorine), aeration to remove volatile compounds, inactivation of metabolically activated toxicants, solid-phase extraction (SPE) of non-polar organic compounds on C-8 columns and subsequent elution of extracted compounds. Control water is also subjected to sample manipulations. Each sample and control water fraction, in which classes of

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compounds have been removed, inactivated, or isolated, is then tested for toxicity. Phase I TIE procedures follow EPA methods described by Mount and Anderson-Carnahan (1988), with minor refinements and modifications as described by Bailey *et al*. (1996). In addition to Phase I TIE manipulations, analyses will be continued on a sample-specific basis to include chemical analyses of test solutions and/or SPE column eluates to assist in the determination of individual compounds responsible for toxicity. The TIE procedures are described briefly below. Phase I TIE procedures are applicable to water samples and porewater extracted from sediments. In the event that the solid phase is found to be toxic, but porewater is not, TIE procedures for sediments will be implemented. These will be selected in consultation with the Project Manger.

Dilution

Each sample and treatment is tested at full strength (100%) and at dilutions to be determined based on results of initial toxicity tests. Testing sample dilutions provides information on the degree of sample toxicity.

Aeration

Samples are aerated for one hour. If results of aeration and/or pH adjustment tests so indicate, additional aeration treatments may be tested, including aeration for one hour at pH 3 (adjusted with HCl), followed by adjustment back to initial pH (with NaOH), and aeration for one hour at pH 11 (adjusted with NaOH), followed by adjustment back to initial pH (with HCl). Toxicity tests are conducted after each treatment solution has been restored to initial pH. Aeration removes volatile compounds. Aeration at low or high pH removes compounds that are more volatile when equilibria are shifted to one or another ionic form.

pH Adjustment

Adjusting sample pH can affect the toxicity of hydrolysable, ionic, acidic, or basic compounds. Sample pH is adjusted to pH 3 by addition of HCl, then held at that pH for 3 hours before returning the sample to initial pH by addition of sodium hydroxide. Sample pH is also adjusted to pH 11 by addition of sodium hydroxide, then held at that pH for 3 hours before returning the sample to initial pH by addition of HCl. Toxicity tests are conducted after the treatment solutions have been restored to initial pH.

EDTA Chelation

Addition of EDTA binds cationic trace metals, such as copper, cadmium, mercury, zinc, lead, nickel, and, to a lesser extent, silver and manganese, resulting in relatively non-toxic metal complexes (Hockett and Mount 1996). Sample pH will be checked after EDTA addition and again prior to distributing samples into test containers. Solutions are adjusted with sodium hydroxide if necessary to return to initial pH.

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Sodium Thiosulfate Addition

Addition of sodium thiosulfate (STS) reduces oxidants, such as chlorine, ozone, chlorine dioxide, mono- and di-chloroamines, bromine, iodide, manganous ions, and certain electrophilic organic chemicals (Mount and Anderson-Carnahan 1988). It also binds some trace metals, such as copper, cadmium, mercury, silver, and to a lesser extent, zinc, lead, and nickel (Hockett and Mount 1996). Addition of STS is used to evaluate toxicity due to oxidants and trace metals.

Solid-Phase Extraction (SPE)

Solid-phase extraction (SPE) through a C-8 SPE column is used to remove a range of non-polar organic compounds from sample solutions, including most pesticides. The SPE columns are then eluted with methanol to allow toxicity testing of non-polar compounds eluted from the column. Two C-8 columns are prepared for each sample and run in parallel using dual peristaltic pumps. Sample is pumped through silicone tubing that has been cleaned by running 25 ml of glass-distilled water and 25 ml of methanol through each tubing apparatus (not through the columns). Columns are prepared by pumping 30 ml of methanol through each, followed by 50 ml of glass-distilled water. Next, laboratory dilution water is pumped through the columns; the first 20 ml is discarded, and subsequent water is kept as the column control solution. Finally, sample is run through each column; the first 20 ml is discarded, and subsequent volumes from each column is collected and combined to provide SPE-treated sample for toxicity testing. Columns are kept wet until all sample water has been passed through. Both columns are then run dry and air-dried with a syringe. 1.5 ml of methanol is added to one column, and air is pumped into the column at 2 ml/min until the column is dry. Eluate is collected in a small solvent-cleaned vial. This step is repeated, pooling the two eluate samples. The second column is archived for chemical analysis, if necessary. Eluate is then delivered into laboratory dilution water to provide the column eluate treatment.

Piperonyl Butoxide Tests

A number of organophosphate pesticides (phosphorothioate compounds such as diazinon, chlorpyrifos, malathion, parathion, methyl parathion and fenthion) require metabolic activation by exposed organisms before they become toxic. These activation reactions consist of oxidative metabolism by the cytochrome P-450 group of enzymes (Durhan *et al*. 1993). This activation can be blocked by compounds such piperonyl butoxide (PBO), thereby reducing or eliminating toxicity due to this class of compounds. In addition, Deanovic *et al*. (1998) found that PBO potentiates the toxicity of certain pesticides (e.g., synthetic pyrethroids), and this may be used as a diagnostic tool to indicate toxicity due to these compounds. PBO is added to test samples to determine whether metabolically activated pesticides are responsible for observed toxicity. PBO controls consist of PBO added to laboratory dilution water in the same concentration as in sample treatments..